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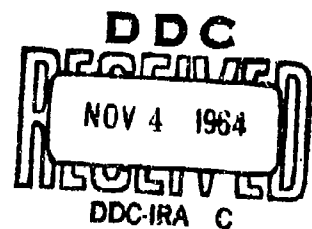
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PURIFICATION AND CHARACTERIZATION OF STAPHYLOCOCCAL ENTEROTOXIN B

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ABSTRACT

A method has been developed for the isolation of enterotoxin B with a purity greater than 99 per cent and in yields of 50 to 60 per cent from cultures of Staphylococcus aureus. The method involves:

(a) Removal of the toxin from the culture with CG-50 resin, partly neutralized at pH 6.4, and elution with 0.5 M phosphate buffer, pH 6.8 in 0.25 M sodium chloride;

(b) Readsorption of the toxin on CG-50, partly neutralized at pH 6.8, followed by elution of the toxin with 0.15 M disodium phosphate; and

(c) Adsorption of the toxin on carboxymethyl cellulose and chromatographic elution with a gradient phosphate buffer 0.02 M to 0.07 M at pH 6.8.

The purified toxin was eluted between 0.035 M and 0.045 M phosphate. Elution of the toxin was followed by the absorbance at 277 millimicrons and by the Oudin serological test. Final preparations were assayed in rhesus monkey.

Ultracentrifugal and electrophoretic studies show that the purified toxin is a single component with a molecular weight of approximately 35,000. Analyses show that the toxin is composed of amino acids only and therefore is a simple protein. The terminal amino acid analyses show one mole glutamic acid as the N-terminal acid and one mole of lysine as the C-terminal acid, indicating that the toxin molecule is composed of a single chain of amino acids. The dose to produce emesis or diarrhea in 50 per cent of the rhesus monkeys is 0.1 to 0.3 microgram per kilogram of monkey weight by intravenous injection and 1 microgram per kilogram by oral feeding.

I. INTRODUCTION

Staphylococcus aureus produces a variety of toxic substances. One of these substances is enterotoxin B, which causes emesis and diarrhea in experimental animals very similar to that caused by enterotoxin A, which is usually found in cases of food poisoning in humans.[1] Bergdoll, Sugiyama, and Dack[2] reported the first significant purification of this toxin, by a combination of acid precipitation, adsorption on alumina, adsorption on IRC-50, and starch electrophoresis. Recently Frea, McCoy and Strong[3] effected a partial purification of this toxin by a combination of alcohol precipitation, filtration on Sephadex, and electrophoresis on Sephadex. Only milligram quantities of toxin were obtained by these methods. This paper describes a method of purification based on chromatographic procedures employing carboxylic acid resins that results in enterotoxin B of higher purity and in higher yields than that obtained by the above methods. The partial physical and chemical characterization of this purified toxin shows that the toxin is a simple protein composed of a single polypeptide chain with a molecular weight of approximately 35,000.

II. METHODS AND RESULTS

The toxin was produced by culturing S. aureus strain S-6 for 18 hours with aeration in a medium containing 1 per cent N-Z-Amine A (Sheffield Farms), 1 per cent protein hydrolyzate powder (Mead Johnson and Co.), 0.001 per cent thiamin, and 0.001 per cent nicotinic acid adjusted to pH 6.5. The fermented culture usually contained between 0.1 and 0.2 mg of toxin per ml, based on Oudin tests. The toxin constituted 0.5 to 1 per cent of the total organic solids in the culture. The culture was passed through a Sharples centrifuge to remove the bulk of the cells. The carboxylic acid exchange resin, used for the first and second steps in the purification, was Amberlite CG-50, 100-200 mesh (Rohm and Haas Co.). This resin was exchanged with sodium at least once and partly neutralized as specified in the purification procedure by suspending the resin in 3 to 4 volumes of 0.05 M monosodium phosphate and titrating to the desired pH with sodium hydroxide. Before use the partially neutralized resin was washed thoroughly with water to remove the buffer. The carboxymethyl cellulose (CM) (Carl Schleicher and Schuell Co.), used in the final step in the purification, was Selectacel Ion Exchange Cellulose, No. 77, Type 20. It was used as purchased after washing thoroughly with water. The linear gradient buffer system used to elute the toxin from the carboxymethyl cellulose was patterned after that described by Mikes.[4] Assays for the toxin were carried out by a modification of the Oudin serological technique.[5] In this technique, the rate of diffusion of the toxin into a tube of agar gel

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containing antisera to the purified toxin serves as a measure of the toxin concentration.[6] These assays require 24 to 72 hours for completion, but are serologically specific for the toxic protein and served as a reasonably accurate means for a quantitative determination of the toxin throughout the purification procedure. The results are expressed as mg of toxin per ml or, with the Kjeldahl nitrogen value, as mg of toxin per mg of nitrogen. Theoretically, when the Oudin assay shows 6.25 mg of toxin per mg of nitrogen, the toxin is pure. However, the variability of the Oudin test is ± 10 per cent and additional tests were necessary to measure small amounts of impurities. Ouchterlony tests,[7] employing antisera from crude toxin preparations in the agar gel, were used for this purpose. Although the serological tests (Oudin and Ouchterlony) do not measure toxicity per se, they correlated well with toxicity tests in monkeys. The absorbance at 277 mμ was used also as a rapid but nonspecific assay to follow the proteins through the chromatographic fractionations. These measurements were made with a Model DU Beckman spectrophotometer. The results are expressed as mg of protein per ml, using an extinction value of 1.4 for 1 mg per ml at 277 mμ in a 1-centimeter cell. The absorbance could not be used as a measure of purity because the contaminating proteins contributed to the absorbance along with the toxin. Final preparations of the toxin were assayed by intravenous injection or feeding to rhesus monkeys* to obtain the specific biological activity characterized by emesis or diarrhea.

Sedimentation studies were carried out on a Model E Spinco ultracentrifuge. Amino acid analyses were carried out on a Phoenix Amino Acid Analyzer by the method of Spackman, Stein, and Moore.[9] Terminal amino acids were determined by N-dinitrophenylation with 2,4-dinitrofluorobenzene followed by acid hydrolysis for the N-terminal acid[10] and hydrazinolysis followed by treatment with benzaldehyde and identification on the automatic amino acid analyzer for the C-terminal acid.[11]

A. PURIFICATION PROCEDURE

The method of purification is outlined in Figure 1 and involves the following steps:

Step 1. The centrifuged culture containing up to 0.2 mg of toxin per ml with an Oudin assay value of 0.04 mg toxin per mg of nitrogen was diluted with two volumes of water and the pH adjusted to 6.4. The toxin was removed from the diluted culture with CG-50 resin partly neutralized to pH 6.4 by stirring for about 30 minutes at room temperature. Two grams of this resin were sufficient to remove the toxin from one liter of diluted culture. Usually about 50 liters of diluted culture, or sufficient culture to isolate

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* In conducting this research the animals were maintained in compliance with the principles established by the National Society for Medical Research."

one gram of purified toxin, were processed at one time. The resin containing the toxin and impurities was filtered into a column (about 3 by 30 cm for resin from 50 liters), washed with one column volume of water, and the toxin fractionally eluted with 0.5 M sodium phosphate at pH 6.8 in 0.25 M sodium chloride. The fractions containing the peak of protein material were selected on the basis of the absorbance at 277 mμ and more precisely selected on the basis of Oudin tests and pooled. At this point the pooled fractions amounted to about one per cent of the culture volume; the Oudin test showed about 4.5 mg of toxin per mg of nitrogen, and the yield was 70 to 80 per cent.

Step 2. The pool of fractions from Step 1 was dialyzed to remove the salts and the toxin was readsorbed on a column of CG-50; this time the CG-50 was partially neutralized at pH 6.8. Twenty grams of resin were used per gram of protein (column about 3 by 30 cm for 20 gm resin). The partial neutralization of the resin at a higher pH reduced the adsorption of the impurities but still allowed complete adsorption of the toxin. Thus, the bulk of the impurities passed through the resin. After the resin was washed with water the toxin was fractionally eluted with 0.15 M disodium phosphate, and the fractions containing the peak of toxin with an Oudin value of about 5 mg of toxin per mg of nitrogen or more were selected and pooled for further purification. At this point the pooled fractions showed an Oudin value between 5.5 and 6 with a purity of 85 to 90 per cent. The yield was 65 to 70 per cent.

Step 3. The combined fractions from Step 2 were dialyzed to bring the salt below 0.01 M and the toxin was adsorbed and chromatographed on a column of carboxymethyl cellulose. Twenty grams of this resin were used per gram of protein as measured by absorbance at 277 mμ. A linear gradient phosphate buffer from 0.02 to 0.07 M, pH 6.8, was passed through the column at a rate of 0.3 ml per minute per square centimeter of area on the column. The purified toxin usually came off in the fractions between 0.035 and 0.045 M, but the exact peak was located by measuring the absorbance at 277 mμ and more accurately evaluated with Oudin tests. The fractions that had Oudin values of 6.2 or greater per mg of nitrogen and that showed only a single antigen-antibody line in the Ouchterlony tests at a level of 0.5 mg of toxin per ml were selected as the purified toxin. These fractions were pooled, dialyzed to remove most of the buffer salts, centrifuged to remove any insoluble material, and freeze-dried. The over-all yield of toxin in the purified state usually amounted to 50 to 60 per cent based on the Oudin assay of the original culture.

B. PHYSICAL AND CHEMICAL CHARACTERIZATION

Table I shows some of the chemical and physical properties of the purified toxin. The freeze-dried protein is a snow-white fluffy powder that is very soluble in water and salt solutions. The dry protein also hydrates

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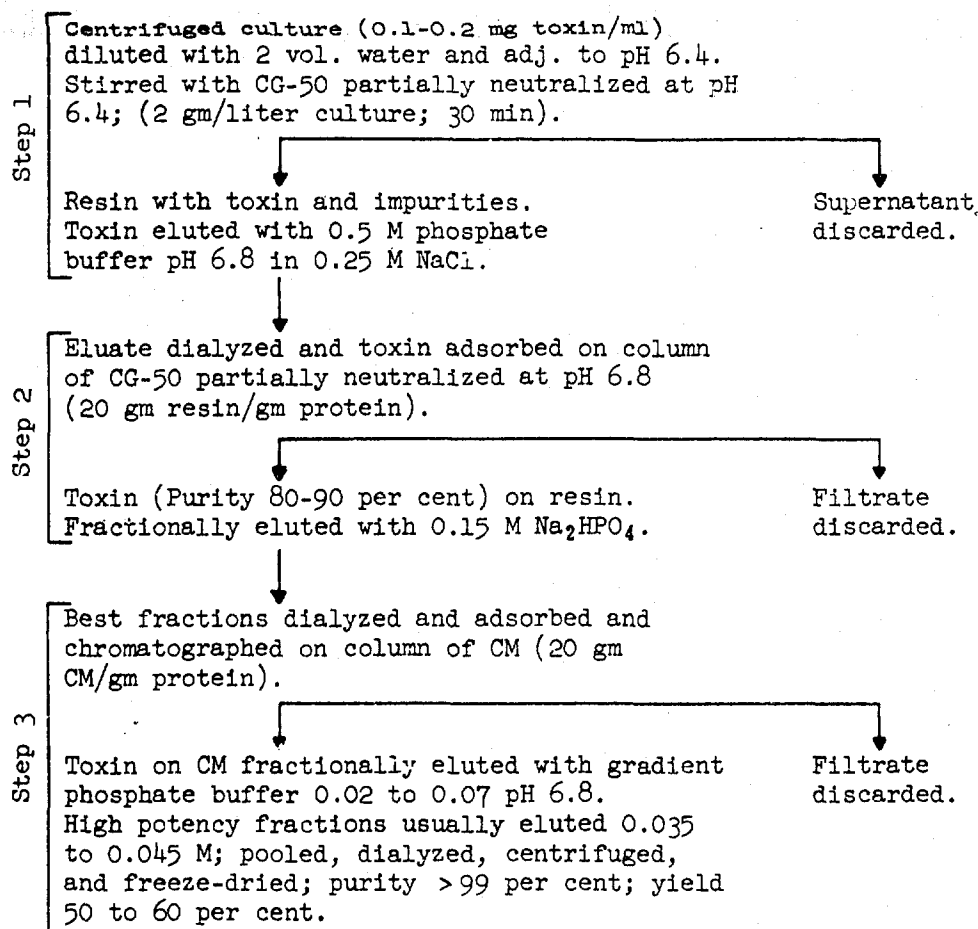
Step 1

Centrifuged culture (0.1-0.2 mg toxin/ml)
diluted with 2 vol. water and adj. to pH 6.4.
Stirred with CG-50 partially neutralized at pH
6.4; (2 gm/liter culture; 30 min).

Resin with toxin and impurities.   Supernatant
Toxin eluted with 0.5 M phosphate   discarded.
buffer pH 6.8 in 0.25 M NaCl.

Step 2

Eluate dialyzed and toxin adsorbed on column
of CG-50 partially neutralized at pH 6.8
(20 gm resin/gm protein).

Toxin (Purity 80-90 per cent) on resin.   Filtrate
Fractionally eluted with 0.15 M Na_2HPO_4.   discarded.

Step 3

Best fractions dialyzed and adsorbed and
chromatographed on column of CM (20 gm
CM/gm protein).

Toxin on CM fractionally eluted with gradient   Filtrate
phosphate buffer 0.02 to 0.07 pH 6.8.   discarded.
High potency fractions usually eluted 0.035
to 0.045 M; pooled, dialyzed, centrifuged,
and freeze-dried; purity > 99 per cent; yield
50 to 60 per cent.

Figure 1.   Scheme for the Purification of Enterotoxin B.

readily when exposed to an atmosphere with a relative humidity of 20 per
cent or more. Tests for carbohydrate, lipid, and nucleic acids were nega-
tive. Tests for α and β lysins, apyrase, and dermonecrotic substances,
normally found in the culture, were negative in the purified preparation.
The Kjeldahl nitrogen content of the protein (free of buffer salts) is 16.1
per cent.

In velocity ultracentrifugation, solutions of the purified enterotoxin
exhibit only a single symmetrical, sedimenting boundary as illustrated by
the representative schlieren curves shown in Figure 2. Analyses of boundary
spreading and of enterotoxin distribution at sedimentation equilibrium show
that the purified material possesses a high degree of homogeneity with

respect to both molecular weight and density. Its partial specific volume (0.743) and infrared spectral absorption are typical of simple proteins. The molecular weight of 35,300 obtained by sedimentation-diffusion is in good agreement with results by approach-to-equilibrium sedimentation. Stability in sedimentation behavior was observed over the pH range 5 to 10.

TABLE I. SOME PROPERTIES OF PURIFIED ENTEROTOXIN B

|	
Appearance	White fluffy powder
Solubility	Very soluble in water and salt solutions
Type of protein	Simple (contains simple amino acids only
Nitrogen content	16.1 per cent
Sedimentation coefficient $(s^o_{20^o,w})$	2.89 S (Single component)
Diffusion coefficient $(D^o_{20^o,w})$	7.72×10^{-7} cm^2 sec^{-1}
Partial specific volume	0.743
Molecular weight	35,300
Electrophoresis (free)	Single component
Isoelectric point	About 8.6
Extinction $(E^{1\%}_{1cm})$	14
Toxicity, ED$_{50}$	0.1 to 0.3 µg/kg (rhesus monkey, IV)
Purity	> 99 per cent
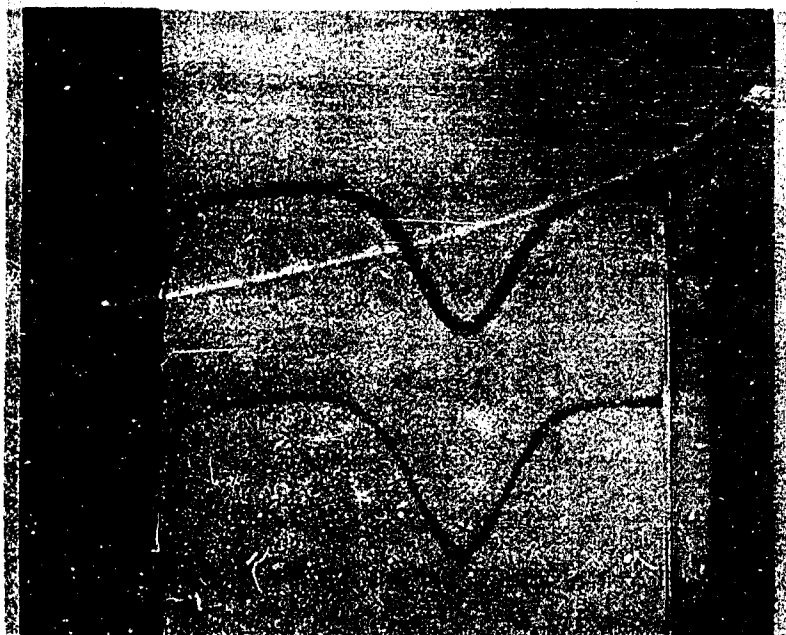
Figure 2. Photograph of Schlieren Sedimentation
Curves of Two Independently Purified
Preparations of Enterotoxin B.

Concentration: 10 mg/ml in 0.05 M
phosphate buffer, pH 6.8. Photo-
graph taken 128 minutes after
reaching full speed, 59,780 rpm.
Sedimentation from left to right;
temperature 20°C; schlieren angle,
60 degrees.

Electrophoretic studies showed the toxin preparation to be a single component with an isoelectric point at about pH 8.6. Deionization of a solution of the toxin on a column of mixed bed resin, MB1, showed an isoionic point at pH 8.55. The toxin has a maximum absorption at 277 mμ with an extinction (E, 1%, 1 cm) of 14. The ratio of the absorption at 260 mμ to that at 277 mμ was 0.47, which confirms other tests showing that very little if any nucleic acid material is present in the preparation.

The amino acid analysis of the toxin is given in Table II. The molecule is composed of 18 different amino acids. No cysteine was found, but 2 half-cystine residues are present. High percentages of aspartic acid and lysine are indicated but, compared with most proteins, the composition of the toxin is not unusual. The recovery of the amino acid residues amounted to 102 per cent and the nitrogen calculated from these residues in addition to the amide nitrogen amounted to 103 per cent. Also, the sulfur in the methionine and half-cystine residues amounted to about 102 per cent of the total sulfur determined by the Parr bomb technique. The complete accountability (within experimental error) of the total nitrogen and sulfur shows that the toxin is composed of amino acids only and therefore is classed as a simple protein. The total of the free acid groups in the aspartic and glutamic acid residues is much greater than that of the free basic groups of lysine, arginine, and histidine, which indicates that the toxin should have an isoelectric point on the acid side of the pH range. The 30 amide groups forming asparagine or glutamine account for the alkaline isoelectric point at about pH 8.6.

The N-terminal amino acid is glutamic acid and the C-terminal acid is lysine. Quantitative estimation of the terminal acids showed 1.1 residues of glutamic acid per mole of protein for the N-terminal acid and 0.73 residues of lysine per mole of protein for the C-terminal acid. These values are within experimental error, under the circumstances of the experiments, for one mole of each terminal acid and are consistent with the representation of the primary structure of the toxin as a single polypeptide chain. No other amino acids were indicated in the analyses for the terminal acids, which lends support to the physical evidence on the high degree of purity of the toxin preparation.

This protein is unusual in many respects. The biological activity characterized by emesis or diarrhea is stable to heating at 100°C for 10 minutes without appreciable loss in activity. Under these conditions the toxin is precipitated from the solution, but when this precipitate is redissolved by cooling and raising the pH of the solution, practically all of the original Oudin activity and sickness-producing properties in monkeys are recovered. The toxin is quite stable over a pH range of 4 to 10 at room temperature. The biological activity is refractory to the action of various enzymes such as trypsin, pepsin, chymotrypsin, and papain.

TABLE II. AMINO ACID COMPOSITION OF STAPHYLOCOCCAL ENTEROTOXIN B

Amino Acid	Amino Acid Residues per 100 gm of Protein, grams	Residues per Molecule to Nearest Integer
Alanine	1.36	7
Amide groups		30
Arginine	2.84	6
Aspartic acid	18.33	56
Cysteine	0.00	0
Half-cystine	0.59	2
Glutamic acid	9.60	26
Glycine	1.91	12
Histidine	2.54	7
Isoleucine	3.49	11
Leucine	6.44	20
Lysine	17.25	47
Methionine	3.09	10
Phenylalanine	5.93	14
Proline	2.22	8
Serine	4.15	17
Threonine	4.56	16
Tryptophan	0.71	2
Tyrosine	10.82	23
Valine	6.10	22

C. BIOLOGICAL EVALUATION

Serological studies on the purified toxin using Oudin and Ouchterlony techniques also indicate a high degree of homogeneity or purity. When the toxin was employed as an antigen at concentrations of 0.5 mg or less per ml and allowed to diffuse into agar gel (Ouchterlony technique) containing antisera against crude preparations of the toxin, only a single line of antigen-antibody formed. However, by using very high concentrations of the toxin (up to 15 mg/ml) in these tests, one or two faint lines appeared, indicating that small amounts of impurity might be present. These lines disappeared completely on a 32-fold dilution of the toxin solution, but the enterotoxin line did not disappear until it had been diluted more than 8000-fold. If a minor antigen is present as an impurity, these data indicate that it constitutes less than one per cent of the preparation. It is possible that the faint lines are artifacts and not impurities. They are not eliminated from the toxin by further chromatography on carboxymethyl cellulose. Also, when the toxin was tested by the slide immunoelectrophoresis

technique, using rabbit, burro, or horse sera, only the one line, due to the enterotoxin, was formed. The toxin therefore is considered to have a purity of 99 per cent or more.

When the toxin was tested in rhesus monkeys, emesis or diarrhea was produced in 50 per cent of the animals (effective dose, ED_{50}) at an intravenous dose of 0.1 to 0.3 microgram per kilogram of monkey weight and at an oral dose of 1 microgram per kilogram.

III. CONCLUSIONS

In conclusion, a chromatographic method has been developed for the isolation of enterotoxin B from S. aureus cultures. Physical, chemical, and biological studies show that the toxin isolated by the chromatographic method is very pure and is a simple protein consisting of a single peptide chain with a molecular weight of approximately 35,000. Many grams of the purified enterotoxin have been prepared and this supply has allowed an extensive research program to be carried out on the characterization and determination of the structure of this protein as well as investigations on the biological effects of the toxin in host animals.

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